Award Number: W81XWH-12-1-0202

TITLE: Genetic Variations in Mitochondria and Prostate Cancer Aggressiveness and Progression in Caucasian and African American Men

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REPORT DATE: September 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
September 2015	Final report	1JUL2012 - 30JUN2015

4. TITLE AND SUBTITLE
Genetic Variations in Mitochondria and Prostate Cancer Aggressiveness and Progression in Caucasian and African American Men

5a. CONTRACT NUMBER
W81XWH-12-1-0202

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
1515 Holcombe Blvd Unit 207
Houston, TX 77030-4009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

It has been suggested that some inherited mtDNA variants could have an adverse effect by increasing the generation of reactive oxygen species (ROS). Besides the sequence variations in mtDNA, the mtDNA CNV might also affect cancer risk by disturbing crosstalk between the mitochondria and the nucleus, and consequently altering nuclear DNA stability. Variability in the mtDNA (sequence and copy number) might be extremely relevant to prostate cancer because oxidative stress has been suggested to play a significant role in prostate cancer carcinogenesis. Even more intriguingly, the geographic and racial polymorphisms of mtDNA might have implications in the racial disparity of prostate cancer because African Americans are at a disproportionately higher risk for many oxidative stress–related medical conditions, including prostate cancer. In this current proposal, we plan to utilize the valuable biospecimens and data collected through North Carolina-Louisiana Prostate Cancer Project (PCaP) to comprehensively study the associations between mtDNA polymorphisms/haplogroups and prostate cancer tumor characteristics at baseline and progression in both CA and AA men. Our hypothesis is that genetic variations (sequence and copy number) in mtDNA are associated with prostate cancer aggressiveness at diagnosis and prostate cancer progression.

15. SUBJECT TERMS
Mitochondrial DNAs prostate cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	39	19b. TELEPHONE NUMBER *(include area code)*
U	UU	U			

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Introduction

The mitochondrial genome is highly polymorphic among individuals and exhibits significant geographic and racial differences. It has been suggested that some inherited mitochondrial DNA (mtDNA) variants could have an adverse effect by increasing the generation of reactive oxygen species (ROS). Besides the sequence variations in mtDNA, the mtDNA CNV might also affect cancer risk by disturbing crosstalk between the mitochondria and the nucleus, and consequently altering nuclear DNA stability. It has been proposed that the copy number of mitochondria per cell reflects the gene-environmental interactions between unknown hereditary factors and the levels of oxidative stress. However, whether the mtDNA CNV could be a predictor of human cancer risk and progression or not remains to be determined. Variability in the mtDNA (sequence and copy number) might be extremely relevant to prostate cancer because oxidative stress has been suggested to play a significant role in prostate cancer carcinogenesis. Considerable effort has been made to discover genetic variation that influences susceptibility to prostate cancer development and progression. However, few have been identified to date. The dilemma might be due to the fact that some of the susceptibility alleles might not reside in nuclear DNA, but in mtDNA. Even more intriguingly, the geographic and racial polymorphisms of mtDNA might have implications in the racial disparity of prostate cancer because African Americans are at a disproportionately higher risk for many oxidative stress–related medical conditions, including prostate cancer. Therefore, the investigation into the role of genetic variation in the mitochondria as a susceptibility factor for prostate cancer could have significant impact on cancer research. In this current proposal, we plan to utilize the valuable biospecimens and data collected through North Carolina-Louisiana Prostate Cancer Project (PCaP) to comprehensively study the associations between mtDNA polymorphisms/haplogroups and prostate cancer tumor characteristics at baseline and progression in both CA and AA men. Our hypothesis is that genetic variations (sequence and copy number) in mtDNA are associated with prostate cancer aggressiveness at diagnosis and prostate cancer progression. The proposed study will represent the first study to address the roles of mtDNA variations in prostate cancer aggressiveness and progression as well as racial difference.

Key words

Prostate cancer, mitochondrial DNA, single nucleotide polymorphism, copy number variation, aggressiveness, racial disparity.

Accomplishment

Task 1: we will evaluate whether genetic variations in mtDNA are associated with aggressive tumor characteristics of prostate cancer at diagnosis and progression of prostate cancer in CA and AA men, and whether the associations are different between CA and AA men. *To achieve this goal, we will estimate the frequencies of mtDNA genetic variants in 2,264 prostate cancer cases (1,139 CAs and 1,125 AAs) from PCaP. The genotypes and haplogroups will be correlated with prostate cancer characteristics at diagnosis for all men. The sub-set of North Carolina PCaP men have been followed for on average 5 years. Five-year biochemical failure (rise in PSA levels) will be the index of prostate cancer 'progression' in this study. We will evaluate the genotypes and haplogroups in relation to the 5-year biochemical failure status in the patients who have at least five* *years of follow-up. In further analyses, we will assess whether the associations are different between AA and CA men while adjusting for confounding.*

We have successfully completed **Task 1**. Briefly, using samples and data from the North Carolina–Louisiana Prostate Cancer Project, we examined cross-sectional associations among epidemiological and clinical characteristics, and 143 mtDNA SNPs, among 864 African American (AA) and 950 Caucasian American (CA) prostate cancer patients. Three SNPs, G9055A (OR=1.65; 95% CI: 1.03–5.42), A10398G (OR=1.44; 95% CI: 1.14–2.92), and T16519C (OR=1.64; 95% CI: 1.06–3.53), were found to be associated with prostate cancer aggressiveness among CA prostate cancer patients. In the haplotype analysis, CA prostate cancer patients classified as haplogroup K show a significant increased risk of developing aggressive prostate cancer (OR: 2.06; 95% CI: 1.24–6.37), whereas those bearing haplogroup U have a significant decreased risk of developing aggressive prostate cancer (OR: 0.67; 95% CI: 0.19–0.89). Unfortunately, we didn't observe any significant association among AA prostate cancer patients. A manuscript based on the results mentioned above is in draft.

In terms of 5-year biochemical failure, currently the number of event is still very small (<15). So we don't have enough statistical power to perform a meaningful analysis. However, we will continue to follow-up the study subjects. Hopefully, with the timing going on, we will observe more events to power up our analysis.

Task 2: we will evaluate whether mtNDA CNVs are associated with aggressive tumor characteristics of prostate cancer at diagnosis and progression of prostate cancer in CA and AA men, and whether the associations are different between CA and AA men. *To achieve this goal, we will quantify the ratio of mtDNA to nuclear DNA, which is an index for the copy number of mitochondria per cell, in 2,264 prostate cancer cases (1,139 CAs and 1,125 AAs) from PCaP. The mtDNA copy number will be correlated with prostate cancer characteristics at diagnosis (aggressiveness status, PSA, stage and grade) and the 5-year biochemical failure status in the patients who have at least five years of follow-up. In further analyses, we will assess whether the associations are different between AA and CA men while adjusting for confounding.*

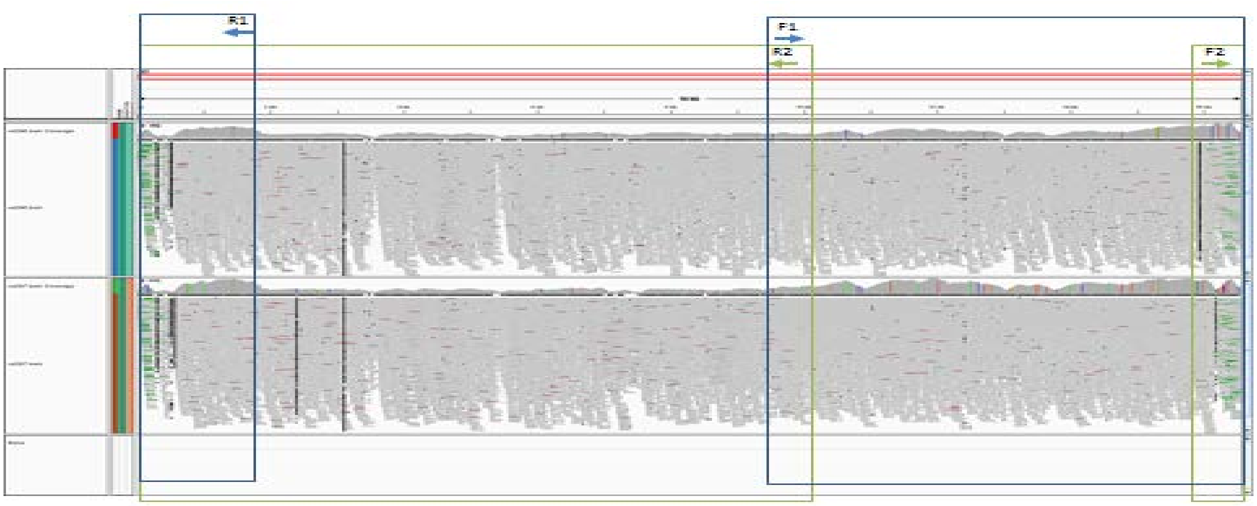
We have successfully completed **Task 2**. A manuscript is attached in **Appendix**. Briefly, using samples and data from the North Carolina–Louisiana Prostate Cancer Project, we examined cross-sectional associations among epidemiological and clinical characteristics, and mtDNA copy number levels in peripheral blood leukocytes, among 864 African American (AA) and 950 Caucasian American (CA) prostate cancer patients. The most significant epidemiological variables related to levels of mtDNA copy number were race, smoking status and body mass index (BMI). The AA patients had significantly higher levels of mtDNA copy number than the CA patients (mean, 0.69 vs 0.62; P<0.001) (**Table 1**). Levels of mtDNA copy number were significantly increased from never, former, to current smokers in CA, AA, and all patients (P for trend: 0.040, 0.003, and <0.001, respectively) (**Table 2**). And levels of mtDNA copy number were significantly decreased as BMI categories increased from normal/under-weight, overweight, to obesity in CA, AA, and all patients (P for trend: 0.026, P<0.001, and P<0.001, respectively). In terms of clinical characteristics, we observed a significant trend of increasing levels of mtDNA copy number when prostate cancer aggressiveness increased from low, intermediate, to high (P for trend=0.004) in AA study subjects (**Table 3**). In further multivariate analysis, we found smoking status, BMI, prostate cancer aggressiveness, age, and race together could explain

17% of variations in mtDNA copy number levels in prostate cancer patients (**Table 4**). In conclusion, we found that levels of mtDNA copy number in leukocytes in prostate cancer patients could be modified by both epidemiological and clinical variables.

In terms of 5-year biochemical failure, currently the number of event is still very small (<15). So we don't have enough statistical power to perform a meaningful analysis. However, we will continue to follow-up the study subjects. Hopefully, with the timing going on, we will observe more events to power up our analysis.

Specific Aim 3: we will explore and perform whole mitochondrial DNA sequencing to identify novel genetic variants in AA and CA prostate cancer patients. *A subset of the study population from PCaP will be selected for sequencing analysis. There will be 25 AA cases with high aggressive prostate tumors, 25 AA cases with low aggressive prostate tumors, 25 CA cases with high aggressive prostate tumors, and 25 CA cases with low aggressive prostate tumors. Any identified novel genetic variant with a minor allele frequency of at least 5% will be further evaluated in terms of their relationship with aggressive tumor characteristics of prostate cancer at diagnosis and progression of prostate cancer in the full cohort of CA and AA PCaP men.*

We have performed mitochondrial DNA sequencing analysis in 25 AA cases with high aggressive prostate tumors, 25 AA cases with low aggressive prostate tumors, 25 CA cases with high aggressive prostate tumors, and 25 CA cases with low aggressive prostate tumors. From each sample library, we obtained 29-102 thousand reads, with an average sequencing depth of 228-2523x and ⩾99% bases covered by at least 30x, except for one sample library with 98.2% bases covered by at least 30x. The PCR duplicate rates in all samples ranged from 0.03-0.27. The mapping rate of the sequenced reads to the reference genome in each sample was 95.4-98.9%; the percent of sequenced reads mapped to mitochondria genome was 88.7-98.5%; and the percent of read pairs with proper orientation was 91.7-97.1%. All were within the expected range, indicating overall good performance of mitochondrion genome sequencing. As an example, we have the sequencing coverage plot of samples ce2b6 and ce2b7 shown in Figure below.



Variant calling and heteroplasmy quantification are performed by the assembleMTgenome.py script implemented in the mtVariantCaller module of MToolBox. The assembleMTgenome.py script takes a SAM file with aligned reads as input, and identified variants are filtered based on both quality score and read depth of supporting bases, with a minimum nucleotide distance from read end for ins/dels calling of 10. Heteroplasmy is then calculated for each variant allele passing all such filters, and sample-specific mitochondrial variant alleles were grouped in three different bins of heteroplasmy: verylowlowheteroplasmy (⩽5%), lowheteroplasmy (>5% and ⩽ 10%), lowheteroplasmy (>10% and <90%) and homoplasmy (⩾90%). For each sample, we observed 0-5 lowheteroplasmy, and 2-67 heteroplasmy. We are currently carrying statistical analysis to associate the low heteroplasmy with clinical and/or epidemiologic factors including ethnicity (both self-reported race and genetic markers), age onset, PSA score, Gleason score, tumor stage, tumor aggressiveness, and etc. Furthermore, bioinformatics analysis will be carried out to annotate the functional impact of heteroplasmy which shows statistical association with clinical and/or epidemiologic factors. We are working on the manuscript.

Because of the low frequency and high diversity of genetic variants identified from the above sequencing analysis, the only way to assess them in the whole cohort is to perform sequencing analysis in the whole cohort, which is cost-prohibitive based on the funding we received. We are applying for additional grant funding in attempt to complete the analysis.

Impact

1. We identified several SNPs and haplotypes associated with prostate cancer aggressiveness among CA prostate cancer patients.
2. We observed a significant racial difference in mtDNA copy number variations.
3. We observed a significant trend of increasing levels of mtDNA copy number when prostate cancer aggressiveness increased in AA study subjects.
4. We discovered a significant amount of new heteroplasmies in both AA and CA prostate cancer patients.

Epidemiological and clinical factors associated with leukocyte mitochondrial DNA copy number among prostate cancer patients in the North Carolina–Louisiana Prostate Cancer Project

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ABSTRACT

Oxidative stress has consistently been linked to prostate carcinogenesis, and mitochondria play an important role in regulating reactive oxygen species generation. However, little is known about the relationship between mitochondrial DNA (mtDNA) copy number in leukocytes and prostate cancer. Identifying factors related to mtDNA copy number in leukocytes among prostate cancer patients may yield insights into mechanisms underlying prostate carcinogenesis and racial difference in prostate cancer. Using samples and data from the North Carolina–Louisiana Prostate Cancer Project, we examined cross-sectional associations among epidemiological and clinical characteristics, and mtDNA copy number levels in peripheral blood leukocytes, among 864 African American (AA) and 950 Caucasian American (CA) prostate cancer patients. The most significant epidemiological variables related to levels of mtDNA copy number were race, smoking status and body mass index (BMI). The AA patients had significantly higher levels of mtDNA copy number than the CA patients (mean, 0.69 vs 0.62; $P<0.001$). Levels of mtDNA copy number were significantly increased from never, former, to current smokers in CA, AA, and all patients (P for trend: 0.040, 0.003, and <0.001, respectively). And levels of mtDNA copy number were significantly decreased as BMI categories increased from normal/under-weight, overweight, to obesity in CA, AA, and all patients (P for trend: 0.026, $P<0.001$, and $P<0.001$, respectively). In terms of clinical characteristics, we observed a significant trend of increasing levels of mtDNA copy number when prostate cancer aggressiveness increased from low, intermediate, to high (P for trend=0.004) in AA study subjects. In further multivariate analysis, we found smoking status, BMI, prostate cancer aggressiveness, age, and race together could explain 17% of variations in mtDNA copy number levels in prostate cancer patients. In

conclusion, we found that levels of mtDNA copy number in leukocytes in prostate cancer patients could be modified by both epidemiological and clinical variables.

Introduction

Mitochondria are organelles in eukaryotic systems that are essential for apoptosis, energy metabolism, and reactive oxygen species (ROS) generation [1]. Compared with nuclear DNA, mitochondrial DNA (mtDNA) is more vulnerable to somatic mutations owing to its lack of histone protection, limited repair capacity, and close proximity to the electron transport chain [2]. The mitochondria are of particular interest to prostate cancer researchers because oxidative stress has been shown to be an important player in prostate carcinogenesis [3, 4]. Investigating the mitochondria may also help elucidate the racial disparity in prostate cancer incidence and mortality rates because the mitochondrial genome is highly polymorphic and shows significant geographic and ethnic variations, with an enormous difference between African mtDNA and European mtDNA [5]. Interestingly, a few genetic variants in mtDNAs have been reported to be associated with several types of cancer, including prostate cancer [6-14].

The mitochondrial genome also shows significant copy number variation [15]. Under normal physiologic conditions, the copy number of mitochondria is genetically controlled and tightly regulated: each cell has multiple mitochondria, and each mitochondrion has 2–10 copies of the mitochondrial genome [15]. Thus, the amount of mtDNA remains relatively stable within the cells. However, it has been shown that mtDNA copy number is influenced by levels of oxidative stress, and these variations in copy number may affect the biologic function of mitochondria [16-18]. In several types of tumors, altered mtDNA copy number is a marker for carcinogenesis [16, 19-28].

More than a dozen molecular epidemiologic studies have been carried out to examine the role of mtDNA copy number in whole blood or leukocytes in the development of certain types of cancer, including renal cell carcinoma, non-Hodgkin lymphoma, breast cancer, esophageal adenocarcinoma, lung cancer, colorectal cancer, and pancreatic cancer [29-42]. Significant but inconsistent associations between mtDNA copy number and cancer development were observed in most of the studies. mtDNA somatic mutations have been widely observed in prostate tumors, and some of the mutations seem to have significant functions in prostate carcinogenesis [4, 22, 43-47]; however, the role of mtDNA copy number in leukocytes in prostate cancer has been rarely studied. To date, the only study of this kind has been a recent analysis of 194 prostate cancer patients from China, in which Zhou et al found that high mtDNA copy number in peripheral blood leukocytes was significantly associated with high Gleason score (P=0.002) and advanced tumor stage (P=0.012) [31].

Using valuable DNA samples and data from the North Carolina-Louisiana Prostate Cancer Project [48], we performed a cross-sectional analysis to investigate the relationship between levels of mtDNA copy number and selected epidemiologic and clinical variables at baseline. We also explored whether the relationship was differed by race.

Materials and Methods

Study population

The patients in the present analysis were originally enrolled in the Prostate Cancer Project, a population-based, cross-sectional study of social, individual-level, and tumor-level causes of racial differences in prostate cancer in the southern United States. All the patients in that study were first diagnosed with histologically confirmed adenocarcinoma of the prostate during the period from July 1, 2004, through August 31, 2009 [48]. Other eligibility requirements for that study were being 40–79 years of age at diagnosis, self-identifying as “African American/Black” (i.e., AA) or “Caucasian American/White” (i.e., CA), being able to complete the study interview in English, and completing all required assessments at the interview. Patients were excluded from that study if they were living in an institution (i.e., nursing home), cognitively impaired or in a severely debilitated physical state, under the influence of alcohol, severely medicated, or apparently psychotic at the time of the interview. Research protocols were approved by the institutional review boards at the University of North Carolina at Chapel Hill (UNC), the Louisiana State University Health Sciences Center (LSUHSC), and the Department of Defense Prostate Cancer Research Program. All research subjects provided written informed consent. Fieldwork was conducted in Louisiana and North Carolina by investigators and staff located at LSUHSC and UNC. During in-home interviews with Prostate Cancer Project research nurses, consenting patients were asked to respond to a series of structured questionnaires designed to solicit information including background characteristics, occupation, family history of prostate cancer, comorbid conditions, healthcare access, prostate cancer diagnosis, prostate-specific antigen (PSA) screening history, and physical activity level.

In addition, medical records were abstracted for information related to the prostate cancer diagnosis. This information included the total serum PSA level (defined as the PSA value obtained closest to and within 1 year prior to the prostate cancer diagnosis date), tumor stage at diagnosis (derived from stage as reported in the medical record) and grade (defined as Gleason score, which is the sum of the primary and secondary Gleason grade values). Cases were classified on the basis of clinical Gleason score, clinical stage, and PSA level at diagnosis as displaying (1) high aggressiveness ([a] Gleason score of ≥8, [b] PSA level of >20 ng/ml, or [c] Gleason score 7 and cT3–cT4), (2) low aggressiveness (Gleason score of <7, cT1–cT2, and PSA level of <10 ng/ml), or (3) intermediate aggressiveness (all other cases). Age at diagnosis was derived from the self-reported date of birth and the date of the diagnostic biopsy as indicated in the medical record and was rounded to the nearest full year.

During the period from July 1, 2004, through August 31, 2009, a total of 2152 patients diagnosed with prostate cancer were identified for enrollment in the Prostate Cancer Project, met all the inclusion criteria given above, and had complete clinical data regarding their prostate cancer's aggressiveness. The 2152 subjects comprised 566 AA men and 568 CA men from Louisiana and 498 AA men and 520 CA men from North Carolina. The participation rates for all subjects enrolled in the Prostate Cancer Project during the same time period were 62% for patients from North Carolina and 68% for patients from Louisiana. The median time between the diagnosis of prostate cancer and the in-home visit with the patient was 118.5 days. Genomic DNA extracted from whole blood collected at the time of each home visit was available for 864 AA men and 950 CA men. These 1814 patients were selected for inclusion in the present study.

Determination of mtDNA copy number by real-time quantitative polymerase chain reaction

The method for determining mtDNA copy number by real-time quantitative polymerase chain reaction is detailed in our previous publication [42]. The method was shown to have high inter-assay reliability. In brief, two pairs of primers were used in the two steps of relative quantification for mtDNA content in leukocytes. One primer pair was used for the amplification of the MT-ND1 gene in mtDNA. Another primer pair was used for the amplification of the single-copy nuclear gene human globulin (HGB). In the first step, the ratio of the mtDNA copy number to the HGB copy number, also known as the mtDNA index, was determined for each sample from standard curves. This ratio is proportional to the mtDNA copy number in each cell and, for each sample, was normalized to a calibrator DNA sample from a healthy control to standardize between different runs. All samples were assayed in duplicate on a 96-well plate with an Applied Biosystems StepOne Plus System. The polymerase chain reactions for MT-ND1 and HGB were performed on separate 96-well plates with the same samples in the same well positions to avoid possible position effects. A standard curve of a diluted reference DNA, one negative control, and one calibrator DNA sample were included in each run. For each standard curve, one reference DNA sample was serially diluted 1:2 to produce a seven-point standard curve between 0.3125 and 20 ng of DNA. The R^2 for each standard curve was 0.99 or greater. Standard deviations for the cycle of threshold value were accepted at 0.25; otherwise, the test was repeated.

Statistical analyses

Statistical analyses were performed using the SPSS statistical package (version 22; SPSS Inc.). Since the mtDNA copy number data were not normally distributed, we performed the analysis using both data with and without log transformation. We found no significant differences in the estimated associations with and without log transformation, and therefore only data without log transformation were presented here. To evaluate the relationships between mtDNA copy number and selected epidemiological and clinical variables, chi-square tests were used for two-level categorical variables, and analysis of variance tests were used for variables with more than two levels. Multivariate linear regression was applied to estimate the association between mtDNA copy number as a continuous variable and selected epidemiological and clinical variables.

Results

Differences in demographic and clinical characteristics at baseline between the CA and AA patients were reported previously [49]. Briefly, compared with the CA patients, the AA patients were significantly younger at diagnosis (mean, 62 vs 64 years old; $P<0.001$); less likely to have a college degree ($P<0.001$), health insurance ($P<0.001$) or treatment with surgery ($P=0.003$); more likely to smoke or have a history of smoking ($P<0.001$); and less likely to meet physical activity recommendations ($P<0.001$). In addition, using SF12 PCS and MCS 50 as the cutoff points, AA patients were more likely than CA patients to have lower mental and physical functioning and lower overall health-related quality of life ($P<0.001$ for both PCS and MCS). In terms of clinical characteristics at baseline, AAs were more likely than CAs to have highly aggressive prostate cancers at diagnosis (19.44% vs 12.84%; $P<0.001$), a high Gleason score (22.29% vs 16.35%; $P<0.001$), and a high PSA level (30.9% vs 20%; $P<0.001$).

We compared mtDNA copy number levels between the AA and CA prostate cancer patients (Table 1). Overall, the AA patients had significantly higher mtDNA copy number levels than the CA patients did (mean, 0.69 vs 0.62; $P<0.001$). When stratified by study location, the significant racial difference was persistent in both LSUHSC and UNC site ($P=0.011$ for LSU and 0.021 for UNC). In further analysis, we investigated the correlation between mtDNA copy number levels and the genetic ancestry. Interestingly, mtDNA copy number was found positively associated with YRI ancestry ($\rho=0.103$, $P<0.001$) and inversely associated with CEU ancestry ($\rho=0.104$, $P<0.001$).

Next, we examined relationships between mtDNA copy number levels and selected epidemiologic variables (Table 2), stratified by race. First, we found that mtDNA copy number levels decreased significantly when the age category increased in AA patients ($P=0.017$). Similar trend was observed in CA patients, too; however, the difference didn't reach statistical significance (P=0.105). For cigarette smoking, we found that mtDNA copy number levels increased significantly from never, former to current smokers in CA and AA patients (P for trend: 0.040 and 0.003, respectively). For BMI, we observed a statistically significant trend of decreasing mtDNA copy number levels with increasing the BMI categories. This trend was evident in CA and AA patients (P for trend: 0.026 and P<0.001, respectively). No significant differences in mtDNA copy number levels were observed between patients grouped by education, family history, health insurance, SF12 PCS, SF12 MCS, or physical activity in CA or AA patients.

In terms of clinical characteristics at baseline (Table 3), when comparing mtDNA copy number levels by prostate cancer aggressiveness, we observed a statistically significant trend of increasing mtDNA copy number levels with increasing the aggressiveness from low, intermediate to high in AA patients (P for trend: 0.004). However, a U-shaped relationship was observed in CA patients: patients with intermediate prostate cancer aggressiveness had lower mtDNA copy number levels than those with low or high prostate cancer aggressiveness (P for trend: 0.008). No significant relationship with mtDNA copy number levels was observed for any tumor stage, any surgery, PSA level or Gleason score.

Finally, we included all epidemiological and clinical variables with p value ≤ 0.15 in the univariate analysis into a multivariate model. The results were presented in Table 4. As expected, the most significant variables included being obese, having high prostate cancer aggressiveness, and being AA. Compared to prostate cancer patients who had under or normal weight, obese individuals had significantly lower mtDNA copy number levels (P<0.001). Compared to those who had low prostate cancer aggressiveness, those who had had high prostate cancer aggressiveness had significantly higher mtDNA copy number levels ($P<0.001$). Compared to CA patients, AA patients had significantly higher mtDNA copy number levels ($P<0.001$). In addition, smoking status (both former and current smokers), overweight, and age were also significantly associated with mtDNA copy number levels. Combined those variables together, about 17.0% of variations in mtDNA copy number levels in prostate cancer patients could be explained.

Discussion

To our knowledge, this is the first study to explore the relationships between epidemiological/clinical variables and leukocyte mtDNA copy number levels among prostate cancer patients. In the study, we found that mtDNA copy number levels were significantly higher in AA prostate cancer patients than CA prostate cancer patients (mean, 0.69 vs 0.62; $P<0.001$). We observed a significant positive relationship between mtDNA copy number levels and prostate cancer aggressiveness in AA patients ($P=0.004$). Additionally, we found smoking status and BMI were correlated with mtDNA copy number levels in both CA and AA patients.

The observed racial difference in mtDNA copy number levels is intriguing. There is evidence that androgen exposure, long known to be a risk factor for prostate cancer, alters the pro-oxidant/anti-oxidant balance of prostate cells, resulting in prostate carcinogenesis [50-53]. The production of ROS by the mitochondria may be influenced by factors such as maternal androgen exposure during gestation [54-56], and time of puberty during adolescence may imprint the prostate to produce more ROS in AA men than in CA men in response to similar levels of androgenic stimulation in adulthood [57]. Intriguingly, compared with the nucleus, mitochondria have reduced DNA repair pathways and are less protected against ROS. Thus, increased ROS production in the prostate in AA men may lead to more mtDNA damage in prostate epithelial cells in AA men than in CA men. To minimize the impact of elevated ROS on mitochondria and to adapt to this unfavorable environment, prostate epithelial cells in AA men may produce more copies of mtDNA than those in CA men. Such an increase in mitochondrial production may explain our observation of a racial difference in mtDNA levels in this study. Several other prostate cancer risk factors, including inflammation and diet, may also affect the production of ROS and subsequently the activity of the prostate cancer cell mitochondria [58]; racial differences in inflammation have been observed in several studies [59, 60]. For example, Wallace et al found prominent immunobiologic differences in prostate tumor tissues between AA and CA men [59]. It is likely that such differences in immunobiology may lead to racial differences in ROS production and thereby in mtDNA copy number.

In a recent study with 194 prostate cancer patients from China, Zhou et al found that high mtDNA copy number levels in peripheral blood leukocytes were significantly associated with high Gleason score and advanced tumor stage but not with serum PSA level (P=0.002, P=0.012, and P=0.544, respectively) [31]. Prostate cancer aggressiveness was not examined in Zhou's study. In contrast, in our study, we did not observe any significant relationships between mtDNA copy number and Gleason score, tumor grade, or PSA level, individually. However, interesting findings were observed for prostate cancer aggressiveness, a collective score based on Gleason score, tumor grade, or PSA level. In AA patients, we observed a significant positive relationship between mtDNA copy number levels and prostate cancer aggressiveness (P for trend: 0.004). In CA patients, a U-shaped relationship was observed: patients with intermediate prostate cancer aggressiveness had lower mtDNA copy number levels than those with low or high prostate cancer aggressiveness (P for trend: 0.008). Although the trends were differed between AA and

CA patients, levels of mtDNA copy number were still highest in patients with high prostate cancer aggressiveness in both CA and AA patients. Thus, our results indirectly confirm the previous findings from Zhou's study [31]. Thus, it is likely that increased levels of mtDNA copy number in patients with prostate cancer aggressiveness may reflect increased tumor burden. Clearly, more research has to be carried out to further clarify the molecular mechanism underlying these changes in mtDNA copy number levels with prostate cancer aggressiveness.

In relation to epidemiologic variables, one of the most consistent observations was a statistically significant trend of increasing mtDNA copy number levels as the smoking status changing from never, former to current). The trends were evident in CA, AA, and all patients. The relationship between mtDNA copy number levels and cigarette smoking was reported previously [39]. In 227 prospectively collected lung cancer cases and 227 matched controls from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, Hosgood et al. reported heavy smokers had higher levels of mtDNA copy number than lighter smokers [39]. Cigarette smoke contains many chemicals that may introduce high levels of ROS in the human body [61]. As such, oxidative stress may play a substantial role in the pathogenesis of smoking-related cancer. Levels of 8-hydroxydeoxyguanosine (8-oxodG), a biomarker for oxidative damage, have been found to be elevated in the lung tissues and peripheral leukocytes of smokers [62]. Similarly, levels of the oxidative stress biomarker F2-isoprostanes, have also been found to be substantially increased in smokers relative to non-smokers [63, 64]. Thus, it is conceivable that current smokers would have higher internal doses of ROS than never and former smokers, supporting our findings that current smokers would have higher levels of mtDNA copy number.

Another consistent observation was a statistically significant trend of decreasing mtDNA copy number levels as the BMI increased (with categories of underweight, normal, overweight, and obese). The trends were evident in CA, AA, and all patients. Adipose tissue is the main source of cytokines and adipokines that increase systemic oxidative stress [65, 66]; thus, obesity may decrease mitochondrial function [67]. The relationship between obesity-related phenotypes and mtDNA copy number levels has been explored in a few studies [68, 69]. Consistent with our findings, Lee et al found that visceral fat area was independently inversely associated with mtDNA copy number levels in 94 healthy young participants ($P<0.01$) [69]. In 144 postmenopausal women, Kim et al found that the levels of leukocyte mtDNA copy number were lower in participants with metabolic syndrome than in those without metabolic syndrome ($P<0.01$) [68].

The strengths of our study include the large sample size of both AA and CA prostate cancer patients, the population-based study design, and the detailed epidemiologic and clinical information available. Our study also has limitations. The main weakness is that the blood samples were not collected before the prostate cancer diagnosis, which prevents us from clarifying the temporal relationship between mtDNA copy number and prostate cancer aggressiveness. In addition, we did not have repeated measures of mtDNA copy number, and a single measurement may not reflect mtDNA copy number over a lifetime. Nevertheless, our study is the first to investigate the epidemiological and clinical factors associated with leukocyte mtDNA copy number in AA and CA prostate cancer patients. Further research is needed to confirm our findings prospectively in other populations and to explore the potential use of mtDNA copy number in prostate cancer risk and clinical outcome prediction.

Funding Sources: This work was supported by the Department of Defense, Prostate Cancer Research Program (W81XWH-12-1-0202).

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Table 1. Levels of mtDNA copy number in prostate cancer cases stratified by race and study site

	CA		AA		
	N	Mean(SD)	N	Mean(SD)	P-value
LSU	499	0.63(0.50)	464	0.69(0.47)	0.011
UNC	451	0.61(0.55)	400	0.68(0.75)	0.021
Total	950	0.62(0.47)	864	0.69(0.53)	<0.001

Table 2. Levels of mtDNA copy number by epidemiological characteristics of prostate cancer cases by race

Characteristics	CA N	CA Mean(SD)	CA P-value	AA N	AA Mean(SD)	AA P-value
Age						
< 57	160	0.63(0.70)		246	0.74(0.43)	
57 - < 63	236	0.62(0.83)		223	0.72(0.47)	
63 - < 69	263	0.60(0.84)		213	0.72(0.52)	
69+	291	0.60(0.56)	0.105	182	0.65(0.55)	0.017
Education						
<8th grade/some high school	94	0.63(0.47)		280	0.67(0.39)	
High school grad/vo-tec	266	0.58(0.67)		287	0.69(0.37)	
Some college/college grad	387	0.60(0.55)		246	0.70(0.62)	
Graduate school/prof. degree	203	0.63(0.47)	0.384	49	0.68(0.43)	0.658
Smoking status						
Never	353	0.59(0.53)		262	0.66(0.49)	
Former	505	0.61(0.49)		398	0.68(0.50)	
Current	92	0.65(0.44)	0.040	202	0.74(0.89)	0.003
Family history(1st degree relative affected)						
No	658	0.60(0.50)		578	0.69(0.51)	
Yes	221	0.62(0.35)	0.557	216	0.70(0.67)	0.624
Body mass index						
Underweight/Normal	165	0.68(0.45)		192	0.74(0.47)	
Overweight	432	0.61(0.59)		342	0.69(0.39)	
Obese	349	0.57(0.54)	0.026	319	0.61(0.61)	<0.001
Any health insurance						
No	39	0.61(0.73)		134	0.68(0.51)	
Yes	907	0.61(0.75)	0.614	721	0.70(0.46)	0.528
SF12pcs						
<=50	536	0.63(0.59)		584	0.71(0.48)	
>50	407	0.58(0.50)	0.131	267	0.67(0.54)	0.655
SF12mcs						
<=50	257	0.62(0.56)		334	0.72(0.40)	
>50	686	0.60(0.55)	0.415	517	0.67(0.54)	0.249
Physical Activity(Min/week)						
Below	189	0.60(0.60)		263	0.68(0.84)	
Meets	172	0.61(0.52)		143	0.69(0.60)	
Exceeds	576	0.61(0.55)	0.71	431	0.69(0.68)	0.675
Physical Activity(METs/week)						
Below	229	0.60(0.62)		321	0.69(0.40)	
Meets	210	0.57(0.46)		156	0.70(0.61)	
Exceeds	498	0.63(0.56)	0.142	360	0.68(0.52)	0.903

Table 3. Levels of mtDNA copy number by clinical characteristics of prostate cancer cases by race

	CA			AA		
	N	Mean(SD)	P-value	N	Mean(SD)	P-value
Prostate cancer aggressiveness						
Low	536	0.62(0.41)		394	0.65(0.51)	
Intermediate	292	0.57(0.58)		302	0.69(0.59)	
High	122	0.65 (0.62)	0.008	168	0.72(0.62)	0.004
Tumor stage						
T1	537	0.62(0.43)		477	0.69(0.72)	
T2	388	0.61(0.51)		358	0.67(0.80)	
T3-T4	15	0.60(0.52)	0.361	14	0.80(0.42)	0.111
Categorized sum of Gleason score						
<7 or 7 with (3+4) pattern	793	0.61(0.54)		666	0.67(0.71)	
≥8 or 7 with (4+3) pattern	155	0.60(0.62)	0.644	191	0.71(0.88)	0.329
Any surgery						
No	379	0.61(0.57)		405	0.68(0.46)	
Yes	571	0.61(0.44)	0.664	459	0.70(0.59)	0.823
PSA						
<4.3	290	0.65(0.48)		179	0.72(0.54)	
≥4.3, <5.5	244	0.61(0.52)		185	0.68(0.40)	
≥5.5,<8.5	226	0.61(0.54)		233	0.68(0.58)	
≥8.5	190	0.58(0.69)	0.314	267	0.69(0.44)	0.626

Table 4. Linear regression predicting mtDNA copy number levels among prostate cancer patients

Characteristics	Estimate (SE)	P Value
Smoking status (Never)		
Former	0.092 (0.044)	0.036
Current	0.185 (0.061)	0.003
BMI (Under/Normal)		
Overweight	-0.098 (0.037)	0.008
Obese	-0.139 (0.038)	<0.001
Aggressiveness (Low)		
Intermediate	0.124 (0.062)	0.081
High	0.207 (0.043)	<0.001
Age	-0.031 (0.039)	0.032
Race (AA vs CA)	0.185 (0.054)	<0.001
R^2	0.170	